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## **Antimetastatic activities of heparins and modified heparins. Experimental evidence**

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*Manuscript*

**Antimetastatic activities of heparins and modified heparins.  
Experimental evidence.**

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### **Abstract**

Heparin is commonly used for prevention or treatment of cancer-associated thromboembolism. Recent clinical evidence indicates that heparin, and low-molecular weight heparin improves survival of cancer patients. Experimental evidence from various animal models consistently supports the ability of heparin to attenuate metastasis. Heparin, apart from its anticoagulant activity contains a variety of biological activities possibly affecting cancer progression, including: inhibition of heparanase, blocking of P- and L-selectin mediated cell adhesion, and inhibition of angiogenesis. The delineation of antimetastatic activity of heparin is in the focus of several ongoing investigations. This review summarizes the current experimental evidence on the biology of heparin as a potential treatment cancer progression.

## **Introduction**

Cancer-associated thrombosis is a frequent complication associated with cancer progression [1]. Heparin and low molecular weight heparin (LMWH) are commonly used for the prevention or treatment of thromboembolic complications in cancer patients [2, 3]. Based on earlier observations linking the anticoagulant therapy with heparin to an improved survival of cancer patients, several prospective clinical trial were launched with the aim to test heparin as a potential anticancer treatment (see reviews [4-6]. Preclinical analysis of heparin provides evidence for its antimetastatic activity in a variety of animal models (see reviews [7, 8]. While the potential of heparin to attenuate metastasis in experimental models is well accepted, the underlying molecular mechanism of heparin action in this process is a topic of ongoing investigations.

Heparin is a complex mixture of natural glycosaminoglycans purified from porcine intestine [9]. These linear polymers are composed of a repetitive disaccharide unit containing a glucosamine residue and glucuronic/iduronic acid [10]. Both units are sulfated and the degree of sulfation of the disaccharide units varies throughout the polymer. Clinical preparation of heparin is a polydisperse mixture of glycosaminoglycans that is adjusted for the anticoagulant activity [9]. However, only a small portion of the heparin preparation actually contains the anticoagulant activity. The binding site of heparin to antithrombin III has been identified as a specifically sulfated pentasaccharide sequence [9]. In addition, multiple subsets of heparin chains were shown to interact with a large number of other molecules including enzymes (e.g. heparanase), cell adhesion molecules (e.g. P- and L-selectin), growth factors and cytokines [9, 11-13]. Specific targeting of any of these biological activities by heparin or LMWH was shown to affect the biology of cancer in certain models. Nevertheless, an identification of the critical biological activity of heparin responsible for the attenuation of metastasis was proven difficult due to the very nature of heparin that is represented by a mixture of

glycosaminoglycans carrying partially overlapping biologically active sites. Nevertheless, the recent use of modified heparins with a characterized “narrower” biological activity *in vivo* points toward the critical role of heparin during metastasis [14-19].

### **Heparin attenuates metastasis in animal models**

Heparin and LMWH were tested in many different animal models for their potential cancer inhibiting activity. Heparins were repeatedly shown to effect cancer progression, while their effect on tumor growth was rather limited [16, 20, 21]. Over the last twenty years, the antimetastatic activity of heparins was primarily observed in animal models of experimental metastasis (Table 1). An overview of studies prior to this period is excellently reviewed elsewhere [7, 8]. The experimental metastasis model does not completely recapitulate the natural process of metastatic spread, thus very early steps of local tumor cell dissemination and intravasation needs to be analyzed in spontaneous metastasis models. Despite many limitations of this experimental approach, the timely defined presence of tumor cells in circulation allows the evaluation of cellular and molecular mechanisms during the hematogenous phase of metastasis. Accordingly the biological effect of heparin during this process can be assessed. A variety of heparin preparations, including unfractionated heparin, LMW heparin and several chemically modified heparins with a reduced or a minimal anticoagulant activity, efficiently reduced metastasis when applied shortly before or after the tail vein injection of tumor cells (Table 1). When heparin was applied twenty four hours before or after the tumor cell injection no effect was observed [22, 23]. Only in one study heparin treatment one day before the tumor cell injection and daily during the first three days form tumor cell injection was reported to attenuate metastasis [24]. However, the attenuation of metastasis in this study may be affected largely by the heparin application at the time of tumor cell injection that was not specified [24]. In general, attenuation of metastasis was

independent of the route of heparin application. Intravenous application of heparin around the time of tumor cell injection always led to reduction of metastasis. Subcutaneous or intraperitoneal application of heparin around the time of tumor cell injection led mostly to attenuation of metastasis [11, 14, 20, 25-27], while in some studies the affectivity of heparin was limited [17, 28-30]. Attenuation of metastasis was repeatedly observed in most of the studies despite a wide range of heparin dosages applied (Table 1). In most studies, the amount of heparin applied exceeded the therapeutic dose used in humans. Recently, the efficacy of heparin to inhibit metastasis was also documented at clinically relevant concentrations [26, 31]. In studies with a limited effect of heparin on metastasis [28], the applied heparin dosage was comparable to studies where attenuation of metastasis was detected (e.g. [25]). However, the limited response to heparin treatment was likely due to differences in time and/or route of heparin application, different amount of heparin applied and possible variation in heparin preparations [17, 28, 29, Niers, 2009 #2178]. Clinically used heparin preparations are adjusted according to their anticoagulant activity. Accordingly, different heparin formulations are not necessarily identical in “other” biological activities responsible for the antimetastatic effect of heparin.

### **Potential mechanisms for heparin affects on metastasis**

Hematogenous metastasis consist of a multistep process in which tumor cells enter the blood circulation, evade immune responses, adhere to the endothelium of distant organs, extravasate and colonize the tissue [32, 33]. The common observation that a single injection of heparin, at the time when tumor cells are still in circulation, attenuates metastasis indicates that inhibition of cellular events during the hematogenous phase profoundly affect this process. This conclusion is further supported by the fact, that the half-life of heparin in blood circulation is relatively short and last for four to six hours [14, 34]. Thus it is unlikely that heparin exerts its

antimetastatic activity through inhibition of angiogenesis that would require a prolonged heparin treatment. Therefore, the antimetastatic activities of heparins were analyzed for their potential to: affect anticoagulation; inhibit heparanase and to block P- and L-selectin in particular.

### **Anticoagulant activity and metastasis**

Unfractionated heparin and LMWH has been in clinical use for decades as an effective inhibitor of fluid phase coagulation by enhancing antithrombin inactivation of factors IIa and Xa. Intravenous injection of tumor cells leads to tumor cell emboli formation resulting in the tumor cell entrapment in the pulmonary vasculature [34-36]. Tumor cell associated activation of the coagulation cascade, often expressed by tumor cells, results in a rapid fibrin deposition detected both by intravascular tumor cells and in the metastatic lesions [36, 37]. Some experimental studies have demonstrated that anticoagulation using antithrombin inhibitor hirudin attenuated metastasis [36, 38]. However, the dose of hirudin used led to an excessive anticoagulation affect that is not compatible with the clinical use. Conversely, a continuous treatment with an oral thrombin inhibitor led to an accelerated pulmonary metastasis [39]. Another anti-metastatic property of heparin, with respect to anticoagulation, is related to the capacity of heparin to release tissue factor pathway inhibitor (TFPI) from vascular endothelium thereby contributing to the antiangiogenic activity of the TF inhibitor [40-42]. The use of non-anticoagulant heparin derivatives in a number of animal models resulted in attenuation of metastasis, further demonstrating that the antimetastatic activity of heparins is independent of anticoagulation [14, 16, 17, 28, 43, 44]. The pentasaccharide fondaparinux, an excellent anticoagulant that potentiates antithrombin III, had no affect on metastasis at clinically tolerable levels [26, 31].

### **Heparanase activity**

Tumor cell invasion is a critical step in the metastatic cascade. The invasiveness of tumor cells is associated with the capacity to degrade extracellular matrix components by producing hydrolytic enzymes such as matrix metalloproteinases or heparanase. Elevated levels of heparanase were associated with cancer progression and poor prognosis in various carcinomas of colon, liver, breast, bladder and also multiple myeloma [45]. Heparanase activity correlated with the metastatic potential of B16 mouse melanoma and Eb lymphoma cells [46, 47]. Heparin treatment inhibited heparanase activity and led to attenuation of metastasis [47, 48]. In a myeloma cell model, enhanced expression of heparanase was shown to induce metastasis [49]. Heparanase expression in myeloma was associated with an enhanced shedding of syndecan-1 and correlated with poor prognosis [50, 51]. Silencing of the heparanase gene led to marked reduction of heparanase enzymatic activity in B16 melanomas that resulted in reduced lung colonization [52]. Heparin derivatives characterized by their ability to inhibit heparanase were evaluated for their anti-metastatic activity in a number of animal models [11, 12, 22]. Since the antimetastatic activity of heparins may be caused by other biological activities, like inhibition cell-cell interaction mediated by P- and L-selectin, heparins were tested for both activities in parallel [14]. P-selectin specific heparin derivatives reduced metastasis of tumor cells regardless of heparanase expression. Heparin derivatives with specific heparanase inhibitory activity reduced the metastatic capacity of tumor cells (e.g. B16 melanoma cells) expressing this enzyme, while showing no affect with tumors without heparanase expression (MC-38 carcinoma cells). These results indicate that inhibition of selectins by heparin probably precedes the heparanase inhibitory activity as observed in the experimental metastasis model.

### **Selectin as mediators of metastasis**



During hematogenous metastasis tumor cells in a blood circulation has to survive and adhere to the vasculature in the target organs. Enhanced platelets-tumor cell emboli formation correlated with metastatic progression [53-55]. Tumor cells covered with platelet aggregates were shown to evade immune responses in the blood circulation [56]. Thus, cell-cell interactions of tumor cell within the vascular environment are critical for metastatic progression. Selectins represent the major group of cell adhesion molecules that were linked to hematogenous metastasis. Selectins are vascular cell adhesion molecules that mediate the initial steps of leukocytes and platelets interactions with the endothelial cells under normal physiological condition [57]. While activation of platelets and endothelial cells lead to a rapid cell surface expression of P-selectin, virtually all populations of leukocytes constitutively express L-selectin. E-selectin expression on the cell surface of endothelial cell is induced by a prolonged activation. In addition to their physiological function, selectin were shown to contribute to metastasis by mediation of cell interaction among tumor cells, platelets, leukocytes and endothelial cells [23, 34, 37, 58-60]. The absence of P-selectin led to attenuation of experimental metastasis in several animal models [23, 58, 59]. A marked reduction of platelet-tumor cell thrombi formation in P-selectin deficient mice was found to be responsible for attenuation of metastasis [34, 58]. An experimental elimination of circulating platelets resulted in reduced metastasis [55], further supporting the role of platelets in this process. Collectively, these observations strongly indicate the pivotal role of P-selectin in facilitation of metastasis through mediation of platelet-tumor cell interactions. In addition, endothelial expression of P-selectin was also reported to enhance metastasis [23]. The bone marrow rescue of lethally irradiated P-selectin deficient mice with the wild type bone marrow led to a marked reduction of metastasis. This observation indicates that early endothelial activation, thereby P-selectin expression on vasculature, contributes to metastasis in these chimeric mice where P-selectin is present on platelets only. E-selectin expression has been also studied for its potential to mediate metastasis. Transgenic overexpression of E-selectin in

the liver increased metastasis to this tissue, indicating the involvement of E-selectin in liver metastasis [60]. In the absence of L-selectin significant attenuation of metastasis was observed in two different mouse models, thus actively implicating leukocytes in facilitation of metastasis [59]. Even a transient inhibition of L-selectin during the intravascular phase of tumor cells led to attenuation of metastasis to the comparable levels as observed in L-selectin deficient mice [37]. Further characterization of leukocytes suggested that myeloid-derived leukocytes through L-selectin mediated interactions mediate the initial steps of metastasis.

Selectins bind to specific glycan structures, which are presented on the cell surfaces of many tumor cells. Virtually all carcinomas express selectin ligands due to an abundant expression of mucins. Carcinoma mucins are carriers of O-glycan structures, including the expression of sialyl Lewis x (sLe<sup>x</sup>) that serves as the core component of a selectin ligand [61]. The positive correlation between sLe<sup>x</sup> expression and poor prognosis was observed in gastric, colon, lung, prostate and breast cancers [62-66]. The presence of carcinoma cells, carrying selectin ligands, in the blood circulation together with the presence vascular selectins makes them the potential mediators of cell interactions leading to metastasis.

### **Heparin reduces metastasis through inhibition of selectins**

Heparin has a variety of biological activities, including the capacity to effectively inhibit P- and L-selectins despite no similarity to natural selectin ligands [13, 67]. The hypothesis that selectins mediate metastasis was explored by a number of laboratories, and the recent evidence confirmed the contribution of P- and L-selectin to metastasis [23, 34, 37]. To address the heparin activity during metastasis, P- and/or L-selectin deficient mice were injected with unfractionated heparin shortly before intravascular injection of either carcinoma or melanoma cells [23, 34, 37, 59]. A single bolus injection of heparin attenuated metastasis in wild type

mice to a similar level as observed in P-selectin deficient mice [23, 34]. Furthermore, single heparin injection in P-selectin deficient mice had no further effect on metastasis [34]. Interestingly, single dose of heparin prior to tumor cell injection further attenuated metastasis in L-selectin deficient mice, indicating that L-selectin involvement in this process is subsequent to P-selectin involvement [59]. This hypothesis was confirmed in another study, where L-selectin mediated recruitment to metastasis cells was observed 9 to 12 hours after tumor cell injection [37]. When heparin was applied six hours after tumor cell injection further attenuation of metastasis was observed in P-selectin deficient mice. Conversely, the application of heparin six hours after tumor cell injections had no further effect on metastasis in L-selectin deficient mice [37]. When heparin was applied either shortly before or six hours after tumor cell injection no further effect on metastasis in P- and L-selectin double deficient mice was observed [59, 68]. Interestingly, repeated injection of high doses of unfractionated heparin led to a prolonged survival of mice, indicating that heparin at such doses might have further antimetastatic activities to selectin inhibition [68]. Finally, extensive characterization of modified non-anticoagulant heparins led to identification of a particular heparin derivative (58% N-acetylated heparin), that contains excellent P-selectin inhibitory activity while preserving minimal heparanase inhibitory activity and reduced growth factor binding activity [14]. This heparin derivative reduced metastasis of mouse colon carcinoma and melanoma cells. Collectively, these findings strongly suggest that heparin inhibits metastasis by interference with P- and L-selectin mediated cell-cell interactions. While the contribution of P-selectin to metastasis is partially characterized, the consequence of L-selectin involvement, thereby leukocytes, remains to be further analyzed.

### **Heparin attenuates metastasis. Published evidence versus a mechanism of action**

Hematogenous metastasis is directly or indirectly responsible for most cancer-related deaths. A summary of experimental evidence on heparin treatment of various cancer cells using a broad range of heparin doses as well as different heparin preparations and derivatives is presented in Table 1. Attenuation of metastasis has been observed when: 1) heparin was applied around the time of intravenous tumor cell injection; 2) the amount of heparin applied was at least at the clinically used concentrations. Only in two studies had heparin treatment no affect on metastasis when applied either one hour after tumor cell injection [29] or one hour before injection [17]. In most of the studies, heparin doses were several times higher than clinically used doses in humans. Considering the higher sensitivity of human P-selectin to heparin inhibition when compared to mouse P-selectin (about 10 fold difference), these findings suggest even better responses to be expected in humans, assuming that P-selectin is one of the primary targets during metastasis [69]. The affectivity of non-anticoagulant heparins to attenuate metastasis clearly separates the anticoagulant activity of heparin from its antimetastatic activity. Considering the transient presence of heparin in blood circulation that usually last for several hours [34], the possible biological activity of heparin on angiogenesis, tumor cell growth and invasiveness mediated by heparanase are rather limited. The accumulated evidence on the anti-metastatic activity of heparin over the large number of studies is compatible with the described role of P- and L-selectin mediated interactions during hematogenous metastasis. Further characterizations of heparins will provide additional insights into the precise mechanism of heparin action during metastasis. However, it might well be that “the sum” of biological activities defined by the very nature of heparin molecules will be the right treatment of cancer progression affecting not only selectins or heparanase, but also a number of other mechanisms potentially critical in a different cancers. Finally, the available evidence from clinical trials [4] strongly supports the future evaluation of heparin as a potential anti-metastatic treatment.

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**Table 1**

Effect of heparins on experimentally induced lung metastasis after the intravenous injection of cancer cells

Tumor type	Heparin dose <sup>a</sup>	Doses schedule <sup>b</sup>	Effect on Metastasis	Reference
M mammary carcinoma	UFH 40 IU/mouse	- 24 h and - 1 h i.p.	Decrease	Lee et al. [27]
M mammary carcinoma	UFH 40 IU/kg	-24 h and - 1 h i.p.	Decrease	Lee et al. [20]
M melanoma	UFH 0.4 mg/mouse	- 20 min i.v.	Decrease	Vlodavsky et al. [22]
		- 1 day i.v.	No effect	
	LMWH 0.4 mg/mouse	- 20 min i.v.	Decrease	
		- 1 day i.v.	No effect	
M melanoma	UFH modified 10 mg/kg	- 10 min & twice a week i.v.	Decrease	Sciumbata et al. [21]
M melanoma	UFH modified 50 mg/kg	- 1 h & once daily for 3 days s.q.	Decrease	Lapierre et al. [28]
	UFH modified 10 mg/kg	- 1 h & once daily for 3 days s.q.	No effect	
M melanoma	LMWH 100 µg/mouse	- 20 min i.v.	Decrease	Miao et al. [11]
R mammary carcinoma	LMWH 3.6 mg/rat	- 20 min i.p.	Decrease	
H colon carcinoma	UFH 100 IU/mouse	- 30 min i.v.	Decrease	Borsig et al. [34]
M colon carcinoma	UFH 100 IU/mouse	- 30 min i.v.	Decrease	Borsig et al. [59]
M melanoma	UFH and UFH modified 0.2-0.5 mg/mouse	Coinjected i.v.	Decrease	Poggi et al. [70]
	UFH and UFH modified 0.1-0.5 mg/mouse	- 1 h i.v.	Decrease	
M melanoma	LAC H or LMWH 1.0 mg/mouse	once daily for 7 days i.v.	Decrease	Ono et al. [44]
M lung carcinoma	LAC H or LMWH 1.0 mg/mouse	once daily for 7 days i.v.	Decrease	
M melanoma	LMWH 10 mg/kg	- 4 h s.q.	Decrease	Amirkhosravi et al. [25]
M melanoma	UFH 12.5 or 60 IU/mouse	- 30 min route not specified	Decrease	Ludwig et al. [23]
	UFH 60 IU/mouse	+ 24 h and then every second day	No effect	
M lung carcinoma	LAC H 0.5-2.0 mg/mouse	- 10 min i.v.	Decrease	Yoshitomi et al [17]
	LAC H 1 mg/mouse	- 1 h s.q.	No effect	
M melanoma	LAC H 1 mg/mouse	- 10 min i.v.	Decrease	
M colon carcinoma	LAC H 1 mg/mouse	- 10 min i.v.	Decrease	
M osteosarcoma	LAC H 1 mg/mouse	- 10 min i.v.	Decrease	
M colon carcinoma	UFH 6.6 IU/mouse	- 30 min s.q.	Decrease	Stevenson et al. [26]
	LMWH 7.3 IU/mouse	- 30 min s.q.	Decrease	
M melanoma	UFH 6.6 IU/mouse	- 30 min s.q.	Decrease	

	LMWH 7.3 IU/mouse	- 30 min s.q.	Decrease	
M melanoma	NAC H 50 mg/kg	- 4 h s.q.	Decrease	Kragh et al. [16]
H melanoma	UFH and LMWH 200 IU/kg	- 1 day & once daily for 3 days i.p.	Decrease	Bereczky et al. [24]
M lung carcinoma	UFH 100 IU/mouse	+ 1 h s.q.	No effect	Szende et al. [29]
	LMWH 38 IU/mouse	+ 1 h s.q.	No effect	
	LMWH 57 IU/mouse	+ 1 h s.q.	Decrease	
M colon carcinoma	UFH 100 IU/mouse	+ 6 h and + 12 h i.v.	Decrease	Laubli et al. [37]
M melanoma	LMWH and NAC LMWH 200 µg/mouse	- 4 h s.q.	Decrease	Mousa et al. [18]
M melanoma	UFH 10-60 IU/mouse	- 30 min i.v.	Decrease	Ludwig et al. [31]
	LMWH 60 IU/mouse	- 30 min i.v.	Decrease	
M melanoma	UFH 0.5-5 mg/kg	- 20 min i.v.	Decrease	Park et al. [19]
	UFH modified 0.5-5 mg/kg	- 20 min i.v.	Decrease	
M squamous cell carcinoma	UFH 0.5-5 mg/kg	- 20 min i.v.	Decrease	
	UFH modified 0.5-5 mg/kg	- 20 min i.v.	Decrease	
H breast carcinoma	UFH 3.3 mg/kg	- 4 h & twice daily s.q.	Decrease	Mellor et al. [71]
H breast carcinoma	LMWH 175 IU/kg	- 4 h & once daily s.q.	Decrease	Harvey et al. [72]
	LMWH 90 IU/kg	- 4 h & once daily for 4 days s.q.	Decrease	
M colon carcinoma	UFH modified 150 µg/mouse	- 10 min i.v.	Decrease	Hostettler et al. [14]
M melanoma	UFH modified 150 µg/mouse	- 10 min i.v.	Decrease	
	UFH modified 300 µg/mouse	- 30 min s.q.	Decrease	
M melanoma	LMWH modified 1-10 mg/kg	- 20 min & once daily orally	Decrease	Lee et al. [73]
	LMWH modified 1 mg/kg	- 20 min i.v. & once daily orally	Decrease	
H lung carcinoma	LMWH modified 10 mg/kg	- 20 min & once daily orally	Decrease	
M melanoma	UFH 15-25 USP/mouse	- 30 min s.q.	Decrease	Yee et al. [74]
M melanoma	LMWH 600IU/kg	prior to tumor cell injection i.p.	No effect	Niers et al. [30]
M melanoma	NAC LMWH 10mg/kg	- 10 min route not specified	Decrease	Niers et al. [39]
M melanoma	UFH and UFH modified 5 mg/kg	- 30 min route not specified	Decrease	Zhang et al. [75]

<sup>a</sup> UFH modified is specifically desulfated; LAC – low anticoagulant, obtained by chemical modification of the heparin; NAC – non-anti-coagulant.

<sup>b</sup> Timing of the heparin administration is given in reference to injection of tumor cells. A negative value refers to heparin injection prior to tumor cell administration. Route of heparin administration: i.v. = intravenous; s.q. = subcutaneous; i.p. = intraperitoneal.

M = mouse, H = human, R = rat